4134–4142 Nucleic Acids Research, 1995, Vol. 23, No. 20

Stability and functional effectiveness of phosphorothioate modified duplex DNA and synthetic 'mini-genes'

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Received June 23, 1995; Revised and Accepted September 18, 1995

ABSTRACT

Several gene transfer techniques that employ 'naked DNA' molecules have recently been developed and numerous gene therapy protocols that make use of 'naked-DNA' have been proposed. We studied the possibility of enhancing the stability of 'naked DNA vectors' and thus also gene transfer and expression efficiencies, by constructing phosphorothioate (PS-) double strand DNA molecules and functional transcription units. We first synthesized short PS-double strand DNA molecules by the annealing of two complementary, 35 nt long, oligonucleotides. The accessibility of DNA modifying enzymes to this molecule was significantly decreased: T4-ligase and kinase activity were respectively reduced up to 1/2 and to 1/6, as compared to the normal phosphodiester molecule. Nucleolytic stability was increased either to purified enzymes (DNase I and Bal31) or to incubations in fresh serum, cell culture medium or in muscle protein extract. Phosphorothioate end-capped complete eukaryotic transcription units (obtained by *Taq* polymerase amplification with PS-primers) were not significantly protected from nucleolytic attack. On the contrary, synthetic transcription units, 'mini genes', obtained by *Taq* amplification with 1, 2 or 3 PS-dNTP substitutions, were resistant to DNase I and Bal31 nucleolytic activity. Transcription efficiency, driven by the T7 promoter, was 96.5, 95 and 33.5% (respectively with 1, 2 or 3 substitutions), as compared to the normal phosphodiester molecules.

INTRODUCTION

In the last few years an increased interest has been focused on DNA and DNA-derivatives as possible therapeutic tools. In particular, the recently raised possibility of directly injecting naked DNA into the muscle of living animals, and the consequent consistent expression of foreign genes, prompted us to investigate the possibility of enhancing gene transfer and expression efficiency. One of the main limitations of this technique is the variable efficiency of gene transfer due to the rapid degradation of exogenous DNA by cellular or serum nucleases both outside and inside muscle cells (1). In order to overcome this obstacle, we studied the possibility of protecting naked double strand (ds) DNA by modifying the phosphorous center which is the target of the nucleolytic attack.

It has successfully and reproducibly been demonstrated that phosphorothioate (PS-) modification of the phosphodiester (PO-) bond protects single strand (ss) oligonucleotides from nuclease degradation (2–4). This approach was applied to antisense oligonucleotides used to block transcription or translation both *in vitro* and *in vivo*, through sequence specific hybridization with targeted genetic segments (5). PS-modification of antisense oligonucleotides maintained, and even enhanced, annealing and blocking effectiveness as compared to PO-oligonucleotides (6). Several reports have demonstrated that PS-capping of oligonucleotides confers a resistance to exonucleases which is comparable to that seen with fully PS-modified oligonucleotides (7). Moreover, thermal denaturation studies have suggested that the introduction of PS-bonds decreases the ability of uniformly PS-modified antisense oligonucleotides to form stable hybrids with RNA because of their marked reduction in T_m (7,8). However, in *in vivo* and intracellular conditions, contrasting results have been obtained on the stability of end PS-capped and fully PS-modified oligonucleotides (7).

On the basis of these observations, we wondered whether a ds phosphorothioate DNA molecule was as nuclease resistant as its ss counterpart and, more importantly, whether such a modified molecule might retain all its functional activity. We developed an experimental protocol, both to investigate the possibility of synthesizing a PS-modified recombinant gene and to test its nuclease resistance and functional activity (Table 1). We first examined the accessibility of PS-modified DNA molecules to

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modification enzymes such as kinase and ligase. The second step consisted of studies on the nuclease resistance conferred by PS-modification to DNA molecules either with purified enzymes, or in conditions mimicking the most common physiological environments (cellular and extracellular). The final aim of our study was to synthesize completely functional and nuclease resistant PS-modified genes. By using *Taq* polymerase, either with phosphorothioated primers or with phosphorothioated dNTPs, we produced end-modified or thoroughly modified genes, respectively. Their nuclease resistance and transcriptional effectiveness were tested *in vitro* and compared to those of unmodified genes.

MATERIALS AND METHODS

Oligonucleotide design and synthesis

For ligase and kinase reactions, a couple of 35 bp oligonucleotides was tested in either the normal PO or the PS form: PS35 or PO35: 5′ CTGCG TACGC ACATA GTGCG ATACC GTCGA CCTCG 3′. cPS35 or cPO35:5′ CGAGG TCGAC GGTAT CGCAC TATGT CGGTA CGCAG 3′.

These oligonucleotides were chosen because they do not show any specific complementarity with any known sequence. As the two oligonucleotides are reciprocally complementary, we used the resulting ds for the nucleolytic degradation tests. In the case of the phosphorothioate oligonucleotides, the modification spans the whole length of the molecules.

For the amplification of the 72 bp mini-gene in the presence of PS-dNTPS, we designed a 5′ primer, T7-C6D, 40 nt long, whose sequence is constituted in its first part (T7) by a T7 promoter specific sequence and in its second half (C6D) by 19 nt corresponding to positions 1751–1769 of human rBAT cDNA (9). The 3′ primer, C5R, was 20 nt long, corresponding to positions 1782–1801 of the same cDNA. T7C6D: 5′ TAATA CGACT CACTA TAGGG AGAGA GCTGG ATGGC ATCGA 3′. C5R: 5′ CTCCA AAATT CAGAA CCACG 3′.

For the amplification of the 3086 bp molecule in the presence of PS-modified primers we chose a couple of primers encompassing a whole eukaryotic functional transcriptional unit: 5′ primer, FT1D: 5′ GACTC ACTAT AGGAA GCTG 3′, corresponding to the border between the prokaryotic sequence and CMV promoter/enhancer region of pCMVE2 (10). 3′ primer, FT1R: 5′ CTAGA GTCGA CCTGC TGCA 3′, corresponding to the very 3′ end of SV40 origin of replication in the same plasmid.

All oligonucleotides were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 392 DNA synthesizer. Synthesis of phosphorothioate modified oligonucleotides was performed using tetraethyltiuram disulfide (TETD, Applied Biosystems) on the same instrument following manufacturer's instructions. All oligonucleotides were deprotected with concentrated ammonia and desalted using NAP 5 (0.2 μmol scale synthesis) or NAP 25 (1 μmol) gel filtration columns (Pharmacia). After deprotection, oligonucleotides were purified using Applied Biosystems OPC cartridges and isolated to length homogeneity by PAGE.

When working with ds oligonucleotides, each oligonucleotide was annealed to its complementary strand at a molar ratio of 1:1 in 1 × SSC for 30 min at 65°C. The resulting ds molecule was electrophoresed on a 4% Metaphor agarose (FMC) gel in 1 × TBE buffer for 1 h at 100 V. DNA was visualized by staining the gel with 0.5 μg/ml ethidium bromide.

For phosphorylation, the 5′ end of each oligonucleotide was labeled using T4 polynucleotide kinase (Amersham) and [γ-^{32}P]ATP (S.A. 3000 Ci/mmol) (Amersham). One microgram (89 pmol) of each oligonucleotide was incubated in the presence of 10 μCi [γ-^{32}P]ATP and 10 U T4-polynucleotide kinase for 1 h at 37°C in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM Spermidine, 0.1 mM EDTA in 20 μl total. Following the reaction, kinase was inactivated by incubation for 10 min at 70°C.

Enzymatic studies

Ligation. A 7 kb blunt-ended DNA restriction fragment was ligated either to ss or ds oligonucleotides. In both cases the 7 kb fragment was previously dephosphorylated at its 5′ ends by calf intestinal alkaline phosphatase (CIAP) (Amersham) as described (11).

The ligation reaction was performed at 16°C overnight, with a 10:1 molar excess of oligonucleotide, using 5 U T4 DNA Ligase (Amersham) in 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG 8000 (Sigma). Following the reaction, ligase was inactivated by heating at 65°C for 15 min.

Double stranded oligonucleotides were ligated to the 7 kb DNA fragment at a molar ratio of 20:1 under the same conditions as the ss oligonucleotides.

All the oligonucleotides used for the ligation reactions had been previously phosphorylated at their 5′ ends as already described. The ds oligonucleotides used for the ligation result from annealing two complementary, previously phosphorylated, oligonucleotides (see above).

The efficiency of the ligation reaction was tested by loading an aliquot of each reaction mixture on a 1% agarose gel that was run for 1 h at 50 V in 1× TBE running buffer and subsequently exposed for autoradiography and densitometric analysis. Efficiency was calculated integrating the densitometric value (performed on a BioRad GS-670 scanning densitometer) of the band intensity with the kinase/labelling efficiency of each oligonucleotide, according to the following formula:

actual ligase efficiency = ligase densitometric value × 1/kinase efficiency

This correction was adopted to utilize equimolar amounts of oligonucleotides in each enzymatic reaction and to guarantee the accurate interpretation of the result.

Nucleases. (i) DNase I. Determination of the rate of DNase I degradation on normal or modified DNA was carried out in 400 mM Tris pH 7.5, 60 mM $MgCl_2$, 20 mM $CaCl_2$. RNase-free DNase I (Stratagene) was used at a ratio of 1 U/μg of DNA. A standard reaction was carried out at 37°C for 0–60 min at a DNA concentration of 20 ng/μl. Twenty microlitre aliquots were removed at specific time-points and the reactions were stopped by adding 20 mM EDTA and subsequent freezing at –20°C. Aliquots were loaded on a 4% Metaphor agarose gel. Electrophoresis was performed in 1× TBE buffer containing 0.5 μg/ml ethidium bromide for 1 h at 100 V.

(ii) Bal31. Bal31 exonuclease assay was performed by adding 0.4 U Bal31 (Amersham)/μg of ds substrate in 20 mM Tris pH 8.00, 600 mM NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 1 mM EDTA at 30°C for 0–60 min. Typically, 4 μg of ds substrate were incubated at a final concentration of 20 ng/μl. Twenty microlitre aliquots were taken at specific times and reactions were stopped

by the addition of 20 mM EDTA pH 8.00 and subsequent freezing. Analysis of degradation products was performed by electrophoresis on a 4% Metaphor agarose gel run in 1× TBE buffer for 1 h at 100 V. Visualization of DNA bands was obtained by ethidium bromide staining (0.5 μg/ml) of the gel.

(iii) Serum nucleases. Determination of the rate of nuclease degradation of the oligonucleotides was carried out in RPMI containing 10% non heat-inactivated fetal calf serum (FCS) (GIBCO). The ds oligonucleotide was added at a final concentration of 20 ng/μl (~1 μM) and incubation was performed at 37°C for 0–6 days. Twenty microlitre aliquots were removed at specific time points and stored at –20°C. Degradation products were analyzed by electrophoresis on a pre-stained (0.5 μg/ml ethidium bromide) 4% Metaphor agarose gel run in 1× TBE buffer for 2 h at 80 V.

Analysis of nuclease susceptibility in serum was carried out in 54% non heat inactivated FCS at a final oligonucleotide concentration of 28 ng/μl at 37°C for 0–24 h. Fourteen microlitre aliquots were taken at the fixed time points and loaded on a 4% Metaphor agarose (FMC) gel, pre-stained with 0.5 μg/ml ethidium bromide. The gel was run in 1× TBE buffer for 2 h at 80 V. When fresh human serum was used, it was obtained from blood of a healthy donor by a 10 min centrifugation at 2000 *g*. Incubation conditions were the same as for FCS.

(iv) Protein extracts. An entire mouse quadriceps was excised, finely minced by a razor, placed into a 1.5 ml microcentrifuge tube containing 100 μl of lysis solution (150 mM NaCl, 10 mM Tris–HCl pH 7.4, 50 mM EDTA) and protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin), gently ground with a pestle and incubated overnight at 4°C. The dissolved muscle suspension was subsequently clarified of nuclei and cellular debris by centrifugation at 7000 *g* for 5 min at 4°C. The resulting supernatant was used as the crude cytoplasmic extract and was stored at –80°C.

Analysis of nuclease susceptibility of ds oligonucleotides in the cytoplasmic protein extract was carried out as described for the experiments performed with RPMI-10% FCS, with the exception that cell extract was used at a final concentration of 73%.

In all degradation assays, densitometric analysis was performed by a BioRad GS-670 scanning densitometer and DNA stability was expressed as a percent of the initial input.

PCR-mediated synthesis of extensively phosphorothioate-modified genes

PCR was performed using 100 ng–2 μg of human genomic DNA. According to the physico-chemical properties of the phosphorothioated DNA (4,13), denaturation was carried out at 94°C for 1 min, annealing at 55°C for 1.5 min and extension at 72°C for 1.5 min for a total number of 50 cycles. The PCR mixture was: 67 mM Tris pH 8.8, 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 2, 25 pmol of each primer, 1 U of *Taq* polymerase (Promega), 100 μM of each phosphorothioated or normal dNTP in a final volume of 100 μl. All amplification reactions were performed on a Perkin Elmer Thermal Cycler 480. For electrophoresis, an aliquot (1/10) was loaded on a 4% Metaphor agarose gel, previously stained with 0.5 μg/ml ethidium bromide.

PCR amplification with PS-modified primers

100 ng of plasmid pCMVE2 (10) were used as a template for each reaction. Amplification conditions were modified (12) to allow the production of a long molecule (3086 bp): denaturation was carried out at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 5 min, for a total number of 25 cycles. PCR reactions were carried out in 100 μl overlaid with 100 μl mineral oil. The final concentration of PCR primers was 0.1 μM each. *Taq* DNA Polymerase was obtained by Promega and used at a concentration of 25 U/ml. The final composition of the PCR buffer was 30 mM Tricine pH 8.4, 2 mM $MgCl_2$, 5 mM β-Mercaptoethanol, 0.01% Gelatin, 0.1% Thesit. All amplification reactions were performed on a Perkin Elmer Thermal Cycler 480.

For electrophoresis, an aliquot (5 μl) was loaded on a 1% agarose gel, and the resulting bands were visualized by staining with ethidium bromide (0.5 μg/ml). Densitometric analysis was performed on a BioRad GS-670 scanning densitometer and amplified DNA yield was expressed as a percent of the normal phosphodiester control.

In vitro transcription

Transcription was carried out on 30 ng of template with 10 U of T7 RNA polymerase (Promega) in a final volume of 10 μl containing 10 mM DTT, 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 5 nmol of each ribonucleoside, 10 U RNAsin and 0.2 μl of [^{35}S]UTP (S.A.> 1000 Ci/mmol) or 0.25 μl of [α-^{32}P] UTP (800 Ci/mmol) at 37°C for 90 min. 1/5 vol (2 μl) of transcription reaction was mixed with 48 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylen cyanol. The mixture was then heated at 95°C for 6 min and chilled on ice for 10 min. An aliquot of 4.5 μl was loaded onto a 6.5% polyacrylamide 10% glycerol non denaturing gel. After electrophoresis the gel was dried and subjected to autoradiography for 12 h. Densitometric analysis was performed on a BioRad GS-670 scanning densitometer: the relative amounts of transcribed molecules were expressed as a percent of the normal control.

Fifty micrograms of salmon sperm DNA were added to each transcription mixture as a carrier for TCA precipitation. Then 1/3 of each transcription reaction was directly spotted on a Whatman GF/C glass microfiber filter that was dried under a lamp and counted in 3 ml of liquid scintillation cocktail (Ultima Gold - Packard) in a LKB 1214 β-counter. The remaining 2/3 were precipitated with ice-cold 10% TCA and then spotted on Whatman GF/C glass microfiber filters; these filters were subsequently washed four times with 3 ml of ice-cold 10% TCA and once with 3 ml of 80% ice-cold ethanol, dried under a lamp and counted in a LKB 1214 β-counter in 3 ml of liquid scintillation cocktail (Ultima Gold - Packard). The efficiency of ribonucleoside incorporation (UTP) was determined by calculating the percent of precipitated acid insoluble material for each reaction.

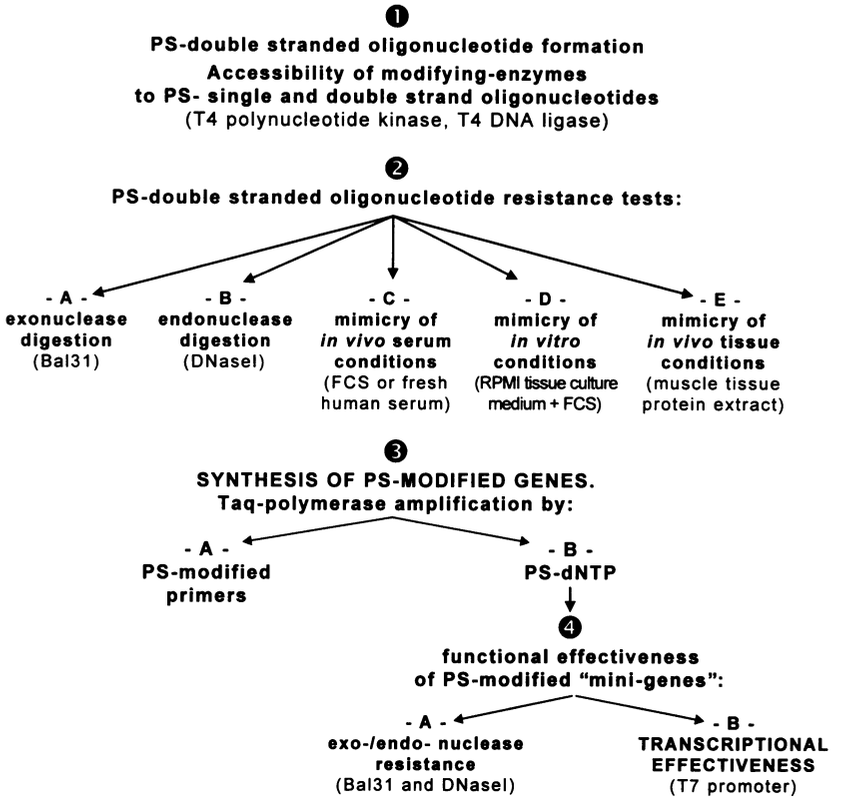
RESULTS

The rationale followed in this research is outlined in Table 1, where every point represents an ideal step towards our ultimate aim: the possibility of producing nuclease-protected functional genes through their partial or total PS-modification.

Accessibility of modifying enzymes to phosphorothioated substrates

Efficiency of T4 polynucleotide kinase, specifically in its forward reaction, was measured as incorporation of [γ-^{32}P]ATP at the 5′ end of 35 bp oligonucleotides. As a substrate, we used either ss

Table 1. Experimental strategy



or ds oligonucleotides, resulting from the *in vitro* annealing of the two complementary ss molecules (see Materials and Methods). Figure 1A shows the results of a phosphorylation reaction performed in the presence of [γ-^{32}P]ATP; the phosphorylated molecules, run on a 4% Metaphor agarose gel, were visualized by autoradiography and analyzed by densitometric scanning: relative [γ-^{32}P]ATP incorporation in PS-modified oligonucleotides was expressed as a percentage of normal phosphodiester controls. In lanes 1 and 2, the labelled molecules resulting, respectively, from 5′ end phosphorilation of phosphorothioate oligonucleotides PS35 and cPS35 are shown. Lanes 3 and 4 represent the corresponding phosphodiester normal oligonucleotides PO35 and cPO35, respectively, labelled at their 5′ end by T4 polynucleotide kinase. Densitometric analysis of these data allowed us to calculate that kinase activity on PS-oligonucleotides (lanes 1 and 2) was reduced from 5- to 12-fold as compared to unmodified controls (lanes 3 and 4). The same loss of activity was observed when kinase was used to phosphorylate ds oligonucleotides, resulting in ~1/6 of control (data not shown).

Similarly, we measured the ability of T4 DNA ligase to ligate either ss or ds PS-modified oligonucleotides to a normal DNA molecule. Oligonucleotides PS35 and cPS35 (see Materials and Methods) were phosphorylated at their 5′ ends by kinase, in the presence of [γ-^{32}P]ATP and were ligated to a 7 kb blunt-ended dephosphorylated DNA fragment. The resulting labelled 7 kb long DNA molecules were evidentiated by autoradiography of a 1% agarose gel. Figure 1B shows the labelled molecules resulting from: ligation with phosphorothioate ss oligonucleotide PS35 (lane 1); ligation with normal ss oligonucleotide PO35 (lane 2); ligation with ds phosphorothioate oligonucleotide PS35-cPS35 (lane 3); ligation with ds normal oligonucleotide PO35-cPO35 (lane 4). Since ligation was performed in the presence of equimolar amounts of DNA molecules, the densitometric data were integrated with the corresponding labeling efficiency. The

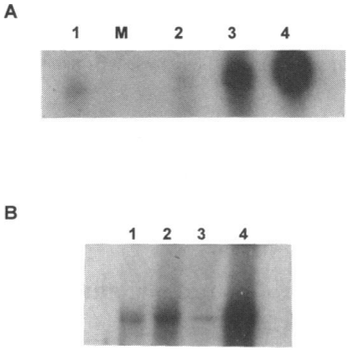


Figure 1. The accessibility of modifying enzymes to PS-modified oligonucleotides. (**A**) Relative efficiency of phosphorilation by T4 polynucleotide kinase on ss substrates differing only for the PS-modification. 1: oligonucleotide PS35; M: molecular weight marker; 2: oligonucleotide cPS35; 3 and 4: control oligonucleotides PO35 and cPO35, respectively. All oligonucleotides were electrophoresed on a 4% Metaphor agarose gel subsequently exposed for autoradiography. (**B**) Ligation of PS-modified oligonucleotides by T4 DNA ligase. Autoradiography of a 1% agarose gel where the products of the ligation reactions between a 7 kb DNA fragment and different oligonucleotides were resolved. 1: ligation with ss oligonucleotide PS35; 2: ligation with normal ss PO35; 3 and 4: ligation with ds PS35-cPS35 or with normal ds PO35-cPO35, respectively.

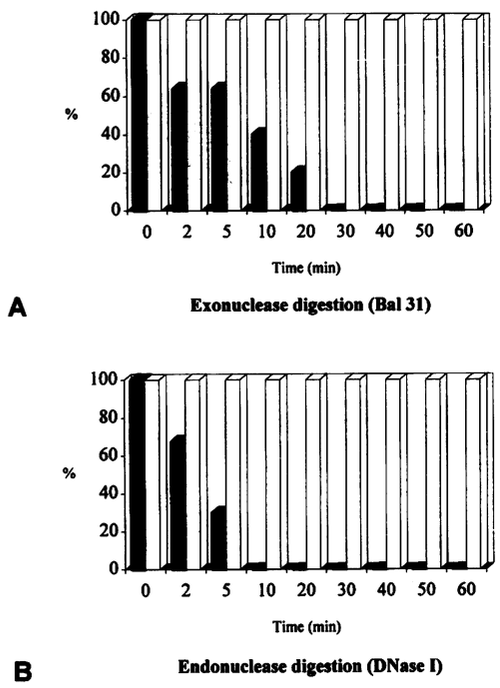


Figure 2. Stability of PS-modified oligonucleotides to degradation by nucleases. (**A**) Time course of *in vitro* incubation of PS-modified ds PS35-cPS35 (grey bars) or of normal PO35-cPO35 (black bars) oligonucleotides with the exonuclease Bal31. (**B**) Time course of the *in vitro* degradation of PS-modified ds PS35-cPS35 (grey bars) or of normal PO35-cPO35 (black bars) oligonucleotides by endonuclease DNase I. After DNA incubations with DNase I or Bal31, equal aliquots of the reactions were directly loaded on a 4% Metaphor agarose gel. The histograms shown represent the results of the densitometric scanning of the ethidium bromide stained DNA bands, where the relative stability is expressed as a percent of the undegraded molecules at time 0. Both the histograms in (A) and (B) represent triplicate sets of experiments.

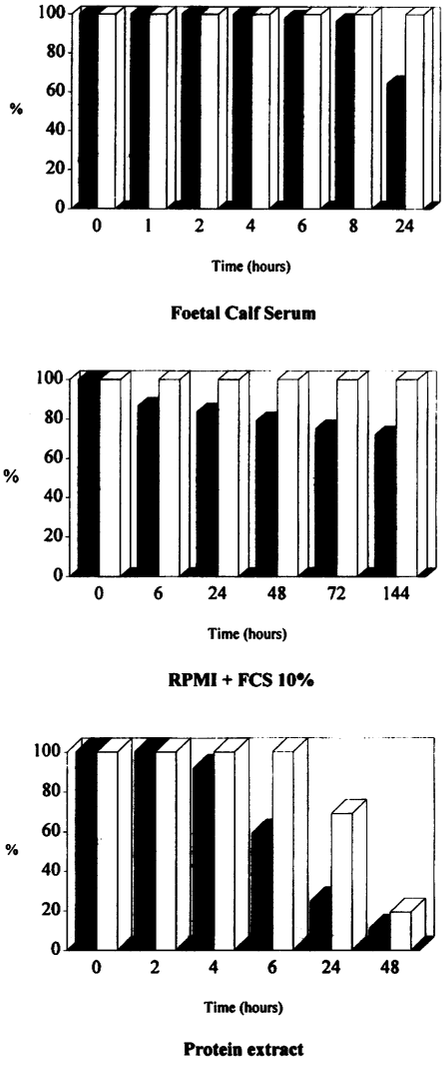


Figure 3. Stability of PS-modified oligonucleotides in the presence of serum or cellular nucleases. (**A**) Degradation time course of normal ds oligonucleotides PO35-cPO35 (black bars) or of PS-modified ds PS35-cPS35 (grey bars) following incubation with non heat inactivated FCS. An identical degradation pattern was reproducibly obtained with the incubation in fresh human serum. (**B**) Nuclease degradation time course of normal ds PO35-cPO35 (black bars) and of PS-modified ds PS35-cPS35 (grey bars) following incubation in a standard tissue culture medium, RPMI-10% FCS. (**C**) Stability time course of unmodified (black bars) ds PO35-cPO35 and phosphorothioate (grey bars) ds PS35-cPS35 incubated in mouse muscle protein extract. After incubations, equal aliquots of the reactions were directly loaded on a 4% Metaphor agarose gel. The histograms shown represent the results of the densitometric scanning of the ethidium bromide stained DNA bands, where the relative stability is expressed as a percent of the undegraded molecules at time 0. Each trial was repeated at least three times.

analysis of the calculated values showed that PS-modification of a substrate, either in the ss or in the ds form, lowers T4 DNA ligase activity to 50% as compared to normal phosphodiester controls.

Stability of phosphorothioated oligonucleotides to degradation by nucleases

Figure 2A represents the results of an *in vitro* incubation of normal (PO35-cPO35) (black bars) and PS-modified (PS35-cPS35) (grey bars) ds oligonucleotides with exonuclease Bal31. The histogram shown is the result of densitometric scanning of a 4% Metaphor gel where the digestion time course points were analyzed. The DNA stability is expressed as a percentage of initial input of each oligonucleotide, and it is worth noting that, while the half life of normal phosphodiester oligonucleotides spans between 5 and 10 min (black bars), PS-modified oligonucleotides show a half life far longer than 60 min (grey bars).

Comparable results are shown in Figure 2B, where DNase I is used as a purified endonuclease. In this case ds PS-modified oligonucleotides (grey bars) appear totally undegraded even after a 60 min incubation, while the half life of normal oligonucleotides is only 3 min (black bars).

After testing purified nucleases, we wondered whether the same degree of protection was conferred to PS-modified molecules in the presence of non-purified serum or cellular nucleases.

Double strand 35 bp oligonucleotides (PS35-cPS35 and PO35-cPO35, see Materials and Methods) were incubated in non heat inactivated FCS and the time course of DNA degradation was analyzed on a 4% Metaphor gel. Experiments were repeated at least 3-fold. The results of the densitometric scanning of one representative gel are summarized in Figure 3A. Although normal phosphodiester oligonucleotides (black bars) also show a

long half life (>24 h) in these conditions, the stability of PS-modified oligonucleotides (grey bars) is always greater, as they appear totally unaffected even after 24 h incubation. An identical degradation pattern was reproducibly obtained when the incubation was performed in fresh human serum. When testing the nuclease resistance of normal and PS-modified oligonucleotides (Fig. 3B) in standard tissue culture medium (RPMI + FCS 10%), we prolonged the time of incubation up to 144 h (6 days). In Figure 3B, black bars represent normal phosphodiester controls which undergo a very slow, but progressive degradation, even if their half life appears to be longer than 6 days; conversely, grey bars represent PS-modified oligonucleotides, which are completely unaffected by serum nucleases, even after 6 days of incubation.

Mouse muscle cell protein extract was used as a source of cellular nucleases for incubation of normal or PS-modified 35 bp ds oligonucleotides. The time course of DNA degradation was followed up to 48 h, and analyzed on a 4% Metaphor gel; the results of the densitometric scanning of the gel are summarized in Figure 3C, where black bars represent normal phosphodiester oligonucleotides and grey bars are phosphorothioate oligonucleotides. As shown in this figure, the half life of normal oligonucleotides is slightly >6 h in these conditions, while PS-modified oligonucleotides still present 69% of undegraded molecules after 24 h of incubation; the half life of these modified molecules is thus calculated ~30 h. Thereafter, PS-modified molecules appear to be at least 5-fold more resistant to cellular nucleases as compared to their normal controls.

Synthesis of PS-modified genes by PCR

3′, 5′ or 3′–5′ PS capping of a long DNA molecule is possible through the ligation of PS-synthesized oligonucleotides. Since ligase activity was significantly reduced, we found that the construction (using several different approaches) of 3′ or 3′–5′ PS-capped ds DNA molecules was severely impaired and not convenient for our purpose. For this reason we tested the possibility of synthesizing PS-modified ds molecules by *Taq* polymerase.

The feasibility of *Taq* polymerase amplification in the presence of phosphorothioated primers was studied by producing a long DNA molecule (3086 bp) that carries only two short PS-modified regions, one on each strand, at the 5′ end of the strand. Figure 4A shows the results of PCR amplification in the presence of PS-modified primers (lane 1) or in the presence of normal phosphodiester primers (lane 2). The use of PS-modified primers in *Taq*-polymerase amplification showed a significant variability among different assays, but altogether only a slight decrease of amplification efficiency. So far, these results demonstrated the possibility of synthesizing end PS-modified long DNA molecules by PCR.

In a second approach, when we forced *Taq* DNA polymerase to utilize PS-dNTPs as synthesis precursors for the amplification of a short DNA molecule (72 bp) representing a synthetic 'mini-gene' (see 'Functional effectiveness of PS-modified genes' for description), we set up slightly modified specific amplification conditions. In fact, it was demonstrated that DNA polymerase I replicates phosphorothioate-modified DNA up to 20-fold more slowly than normal phosphodiester molecules (13). For this reason, we prolonged the length of the polymerization step, as compared to standard conditions and we increased the number of

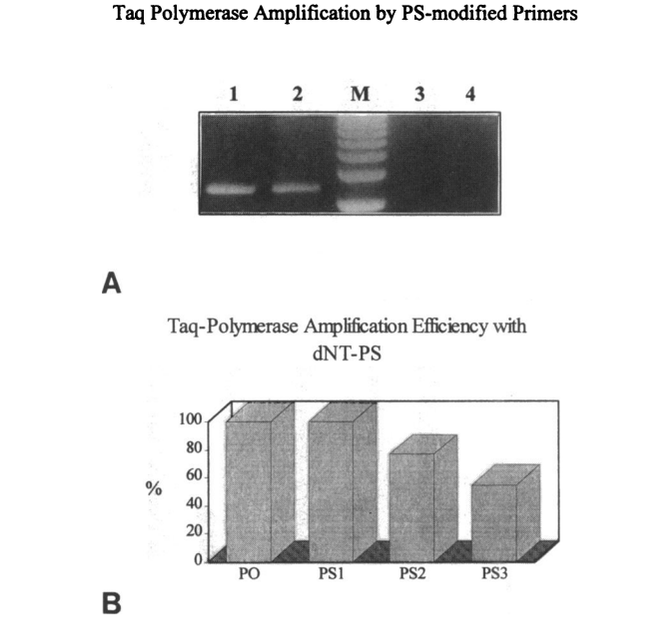


Figure 4. Synthesis of PS-modified genes. (**A**) *Taq* polymerase amplification by PS-modified primers of a 3086 bp DNA region in the presence of PS-modified (lane 1) or normal (lane 2) primers. M: molecular weight marker, 1 kb ladder; 3 and 4: PCR negative controls. (**B**) Histogram representing the relative amplification efficiency of *Taq* polymerase in the presence of one (PS-dATP in PS1), two (PS-dATP + PS-dCTP in PS2) or three (PS-dATP +PS-dCTP + PS-dGTP in PS3) PS-modified precursors, as compared to standard conditions (four normal precursors, in PO). The resulting amplified molecules were 72 bp long and were analyzed on a 4% Metaphor agarose gel which was scanned by densitometry of the ethidium bromide stained DNA bands. Values obtained were reported as percent of amplification efficiency compared to 100% yield obtained under standard conditions (PO). The histogram shown represent duplicate sets of experiments.

cycles up to 50. Amplification reactions were performed in the presence of one (PS-dATP), two (PS-dATP + PS-dCTP) or three (PS-dATP + PS-dCTP + PS-dGTP) PS-modified precursors. As shown in Figure 4B, when only one PS-modified precursor is used (PS1) *Taq* polymerase amplification efficiency is apparently the same as in control amplification with phosphodiester nucleotides (PO). In PS2, where two precursors are PS-modified, *Taq* polymerase works at 76.5% efficiency as compared to the standard control (PO) and the efficiency is decreased to 54.5% when three precursors are PS-modified (PS3).

Nuclease resistance of *in vitro* synthesized PS-modified genes

The same molecules, PO, PS1 and PS2, produced by PCR amplification employing PS-dNTPs were tested in nuclease resistance assays, performed with the exonuclease Bal31 and the endonuclease DNase I. Figure 5A is a schematic representation of Bal31 digestion time course: it is clear that the half life of PS-modified molecules (PS1 and PS2; light and dark grey bars, respectively) is significantly higher as compared to the normal control (PO; black bars). By densitometric scanning of ethidium bromide stained electrophoretic bands, we were able to determine that the half life of PS1 and PS2 is ~30 min, while PO has a half life of ~15 min.

As shown in Figure 5B, the resistance of PS-modified molecules to endonuclease DNase I is even higher than to Bal31: PS1 and PS2 (light and dark grey bars, respectively) are still

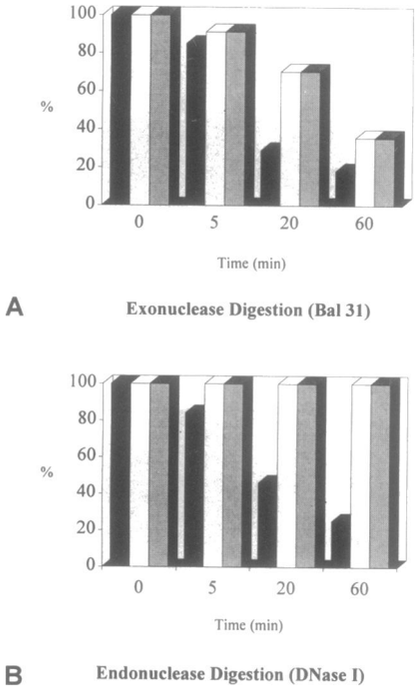


Figure 5. Nuclease resistance of *in vitro* synthesized PS-modified mini-genes. (**A**) Exonuclease Bal31 digestion time course of increasingly PS-modified DNA molecules. (**B**) Endonuclease DNase I digestion time course of increasingly PS-modified DNA molecules. The histograms shown represent the result of the densitometric scanning of the 4% Metaphor agarose gels where the digestion time courses were analyzed: the stability is expressed as the amount of intact material as compared to time 0 (100% stability). PO (black bars), PS1 (light grey bars) and PS2 (dark grey bars) contain, respectively, 0, one or two PS-dNTPs substitutions (see text and Figure 4 for description). Each histogram represents triplicate sets of experiments.

totally undegraded after 60 min of incubation, while the half life of the normal control (PO: black bars) is slightly shorter than 20 min and, after 60 min of incubation, the portion of undegraded molecules drops to 25% of total material.

On the contrary, as expected, DNase I or Bal31 degradation assays performed in the presence of 5′ PS-capped 3086 bp molecules (see section 3) did not show any significant protection of the end modified molecules from the nucleolytic attack, with respect to the unmodified DNA controls (data not shown).

Functional effectiveness of PS-modified genes: *in vitro* transcription

PCR amplification with PS-dNTPs was designed to obtain a synthetic 'mini gene' (72 bp) able to be transcribed *in vitro*. This 'mini gene' was constituted of the T7 promoter region at its 5′ end (included as a flushing end in the 5′ primer sequence) and of a short coding region of rBAT gene (9). As shown in Figure 6, the transcription efficiency of these increasingly modified templates was tested *in vitro* by T7 RNA polymerase using [α-^{32}P] UTP to produce ^{32}P-labelled RNAs. Transcripts were visualized by autoradiography of the corresponding gel and the relative intensity of each RNA band, compared to the band in lane 1 (PO: transcript from a normal phosphodiester template), was quantified by densitometric scanning. Results are summarized in the upper panel in Figure 6. Efficiency of transcription remains

	PS-dNTP	% PS-dNTPs/ total	% PS-dNTPs in T7 promoter	% transcription efficiency
PO	0	0	0	100
PS1	A	14	24	96.5
PS2	A + C	26	43	95
PS3	A + C + G	37.5	62	33.5

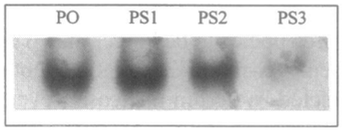


Figure 6. *In vitro* transcription of a synthetic PS-modified mini-gene by T7 RNA polymerase. Autoradiography of T7 RNA polymerase transcription products from normal (PO) or increasingly PS-modified (PS1, PS2, PS3; see text and Fig. 4) templates. Transcription was performed in the presence of [α-^{32}P]UTP, thus yielding ^{32}P-labelled RNAs which were electrophoresed and exposed for autoradiography. Results of the densitometric scanning of autoradiography are described in the upper panel where the type of substitution (PS-dNTP), the percent of PS-modified bases with respect to the whole length of the molecule (% PS-dNTPs/total) and the percent of PS-modified bases in the T7 promoter area (% PS-dNTPs in T7 promoter) are indicated, together with the relative trancription efficiency (% transcription efficiency).

comparable to the normal control when one or two PS-dNTPs are present in the templates (96.5% for PS1 and 95% for PS2) but it drops to 33.5% when three precursors are PS-modified (PS3) and, more importantly, 62% of the bases forming the promoter region are phosphorothioated. These data were reproducibly confirmed also by TCA precipitation of the transcription reactions, where the amount of acid insoluble material for each reaction was determined by liquid scintillation counting. In this assay, only RNA transcribed from PS3 showed a significant decrease in [α-^{32}P]UTP incorporation, suggesting that T7 RNA polymerase efficiency on this template was reduced to at least 50% as compared to PO, PS1 and PS2.

In addition, RNA transcripts were analyzed for their molecular integrity and correct coding sequence. No alterations were found either in the length or in the sequence.

DISCUSSION

Gene transfer is currently the basis of many emerging therapeutic strategies. In the last few years various gene transfer techniques that employ 'naked' DNA molecules (14–17) have been developed and several gene therapy protocols that make use of 'naked-DNA' as bio-pharmaceutical product or protein-factory have been proposed (18–21). The general requirements of all 'naked' DNA gene therapy strategies are essentially a highly efficient delivery of the DNA molecules into the target cells and a consequent high level of expression of the encoded therapeutic gene product; at the same time, it is evident that the efficacy of a gene therapy protocol also depends on the persistence of the delivered DNA, and on its long-lasting expression (22–25). While many technical improvements have been introduced in the gene delivery protocols, and new efficient expression vectors are constantly under development to allow the massive production of very stable mRNAs which are easily translated into their therapeutic protein products, the problem of nuclease degradation

has not been systematically approached. However, nucleolytic degradation appears to be a protection mechanism widely diffused in all biological media, as well as in the intracellular environment. The demonstration that serum is particularly rich in 3′ exonuclease activity (7,8) and that the incubation of 'naked' DNA in seminal plasma or in epydidimal fluid results in a fast nucleolytic digestion (26,27), together with several indications of the intracellular presence of exo- and endo-nucleases (7), led us to investigate the possibility of protecting the exogenously introduced DNA by some chemical modification.

Modifications of the phosphodiester backbone are already known to enhance the nuclease stability of ss oligonucleotides (2,8,29). In particular, protection of the internucleotide linkage by phosphorothioate substitution results in an increased resistance to both exo- and endo-nucleases, either when the PS-modification spans the whole length of the oligonucleotide, or when only the 5′ and the 3′ termini are 'PS-capped' (3,4,7). However, the majority of the studies on PS-modifications deal with short, ss DNA molecules, usually employed as antisense inhibitors of gene expression (4–8). In this study we shifted the attention to the possibility of producing long, ds, biologically functional DNA molecules to use for *in vivo* gene transfer, in the attempt to confer them a significantly enhanced nuclease stability.

The first aim of our experimental strategy was to assess the possibility of manipulation of PS-substituted DNA molecules by the modification enzymes classically used in current genetic engineering protocols. As phosphorilation and ligation are prerequisite steps in most DNA manipulation protocols, we chose to test kinase and ligase accessibility to PS-modified molecules. In accordance with data reported by others (28), we found a great difference in kinase activity between normal and phosphorothioate substrates, resulting in a sharp reduction of up to 1/12 as compared to normal control. Comparably, a severe impairment of ligase activity was observed when one of the molecules to be ligated was a PS-substituted one.

However, even with reduced efficiency, the enzymatic manipulation of PS-modified molecules is still possible. If these molecules are also nuclease resistant *in vitro* and, more importantly, *in vivo,* they could represent good candidates for the synthesis of PS-modified, nuclease-resistant, genes. Thus, our efforts were directed at reproducing the possible nucleolytic activities known to be common *in vivo* (7,8): the endonuclease DNase I and the exonuclease Bal31 were used as purified enzymes, while a closer mimicry of the physiological environments of gene transfer was obtained by challenging PS-modified DNA with serum, cell culture medium or with muscle cell cytoplasmic protein extract. These assays demonstrated a greatly enhanced nuclease stability of PS-modified ds molecules in conditions that resemble the plasma circulation of a living animal and the muscle cell intracellular environment, where the 'naked DNA' intramuscularly injected for gene therapy must face the physiological nucleolytic attack.

In order to exploit the reported nuclease stability of PS-modified DNA, we asked whether this modification was also able to confer protection to long DNA molecules, even if the PS-substitution doesn't span the whole molecule length. *Taq* DNA polymerase was used with the aim of synthesizing these partially modified genes, and its polymerization efficiency was tested either in the presence of phosphorothioated primers or with PS-dNTPs as synthesis precursors. *Taq* polymerase efficiency was only slightly affected by the use of PS-primers, but the resulting amplified molecules, that had only a minimal, ss, 5′-end PS-modification, did not show any enhanced nuclease stability: degradation assays in the presence of DNase I or Bal31 showed no significant differences between these end-modified molecules and their normal DNA counterparts (data not shown). Apparently, a more extensive modification is required to confer significant nuclease protection to long DNA molecules, as demonstrated by another approach, where *Taq* amplification is performed using PS-dNTPs as synthesis precursors. While *Taq* polymerization efficiency is still comparable to the normal controls if one (PS-dATP) or two (PS-dATP + PS-dCTP) precursors are employed, the nuclease stability of the resulting molecules is significantly higher than that of the normal phosphodiester control either when an exonuclease, Bal31, is used and, particularly, when an endonuclease, DNase I, is employed.

These data indicate that it is possible to synthesize *in vitro* a thio-substituted, nuclease resistant DNA molecule. When this molecule consists of a short but complete transcriptional unit the assessment of its transcriptional functionality demonstrates the possibility of synthesizing *in vitro* a functional 'mini-gene'. Thus, the molecules resulting from the amplification with PS precursors, encompassing the entire T7 promoter and a short sequence to be transcribed, were used as templates in an *in vitro* transcription assay by T7 RNA polymerase. The results we obtained clearly indicate that when the template molecules are partially modified in one (dATP) or two (dATP + dCTP) nucleotides, their transcription rate is still very high and comparable to that of the normal control. On the other hand, these molecules are significantly more resistant to nucleolytic degradation as compared to their normal phosphodiester counterparts. Eventual alterations, which might impair the protein product, could be introduced only during the course of transcription, due to possible stops of the T7-RNA polymerase (possible incorrect recognition of a PS-base?) or mistakes in transcription (possible misinterpretation of a PS-nucleotide?). Since RNA transcripts are synthesized using normal, not phosphorothioated, ribonucleotides, translation is not expected to be affected. We tested RNA transcripts and did not find any alterations either in the length or in the sequence. For these reasons, such an *in vitro* synthesized modified molecule may represent a functional, nuclease protected 'mini-gene' to be considered as a prototype construct for gene transfer experiments.

In conclusion, in accordance with previous findings on ss short oligonucleotides (2,4,6–8,29), we report that a thorough PS-modification is able to confer a strong protection from nucleolytic degradation also to ds DNA molecules significantly longer than a simple oligonucleotide. Moreover, we demonstrate that such modified molecules can be synthesized *in vitro* by PCR and, above all, that they can be transcribed, thus representing synthetic functional transcription units. However, we extended our observations by demonstrating that a 5′ end-capping PS-modification of a long DNA molecule is not sufficient for its nuclease protection. We also confirm previous reports about the low accessibility of modifying enzymes to phosphorothioate DNA molecules (28), which implies several manipulation difficulties.

Our results, together with a previously reported observation showing that the persistent presence of PS-DNA *in vivo* can elicit an immune response in injected mice (30), suggest that phosphorothioate modification might represent a way of protecting injected DNA molecules for gene transfer. Further studies are, however, needed to circumvent the problems arising from the

possible immunogenicity, handling difficulties and enzyme accessibility of the modified molecules.

ACKNOWLEDGEMENTS

This research was partially supported by Grants from the Italian National Research Council, Special Projects BTBS to VMF and FATMA to MGF, and from the Italian Ministry of University and Scientific Research Project (60% and 40%). We are also grateful to the IRCCS 'Casa Sollievo della Sofferenza' for generous financial support.

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